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Research article

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## Identification of a distinct class of cytoskeleton-associated mRNAs using microarray technology

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### Abstract

**Background:** Interactions between mRNA and the cytoskeleton are critical for the localization of a number of transcripts in eukaryotic somatic cells. To characterize additional transcripts that may be subject to this form of regulation, we developed a two-step approach that utilizes biochemical fractionation of cells to isolate transcripts from different subcellular compartments followed by microarray analysis to examine and compare these subpopulations of transcripts in a massively-parallel manner.

**Results:** Using this approach, mRNA was extracted from the cytoskeleton-rich and the cytosolic fractions of the promyelocytic HL-60 cell line. We identify a subset of 22 transcripts that are significantly enriched in the cytoskeleton-associated population. The majority of these encode structural proteins and/or proteins known to interact with elements of the cytoskeleton. Localization required an intact actin cytoskeleton and was largely conserved upon differentiation of precursor HL-60 cells to a macrophage-like phenotype.

**Conclusions:** We conclude that the association of transcripts with the actin cytoskeleton in somatic cells may be a critical post-transcriptional regulatory event that controls a larger class of genes than has previously been recognized.

### Background

Subcellular localization of mRNAs represents a fundamental mechanism for post-transcriptional control of cell and tissue function. Examples include the polarized localization of oocyte mRNAs which is crucial for the establishment axis formation in the embryo [1–3], the targeting of specific mRNAs to the synapses of nerve cells [4,5], centrosomal segregation of mRNAs in the mollusk embryo which results in asymmetric inheritance [6] and the localization of  $\beta$ -actin mRNA to sites of active actin polymeri-

zation at the leading edge of motile fibroblasts [7,8]. In each of these cases, mRNA targeting is mediated by the cytoskeleton. For example, growth factor-induced localization of actin mRNA to the leading edge of fibroblasts is a dynamic, regulated process requiring actomyosin interactions and activation of the RhoGTPase pathway, as well as specific signal sequences in the 3' UTR of the message [9–12]. Moreover, disruption of the actin cytoskeleton using pharmacological agents also blocks the association of  $\beta$ -actin mRNA with microfilaments and prevents

localization of the transcript to the cell periphery [7,13]. Mechanical forces exerted on cell surface integrin receptors, which anchor the actin cytoskeleton to the extracellular matrix, also produce changes in the localization of poly(A) mRNA and ribosomal proteins inside the cell [14]. Importantly, the localization of transcripts can serve as a key regulatory step in gene expression as inhibition of mRNA targeting can significantly impact cell function. For example, replacing the 3' UTR of the mRNA for the intermediate filament protein, vimentin, with the  $\beta$ -actin 3'UTR sequence results in mislocalization of vimentin transcript, altered fibroblast morphology, and impaired motility [15].

To date, only a relatively small number of gene transcripts are known to target to the cytoskeleton and all of these have been found empirically. *In situ* hybridization technologies and microinjection of fluorescently labeled RNAs have greatly enhanced our ability to observe intracellular RNA localization with high spatial resolution. However, the number of RNA species that may be simultaneously observed using these approaches is usually limited to only one transcript at a time. We hypothesized that association of mRNAs with the cytoskeleton may be a more widespread post-transcriptional regulatory mechanism that extends to a larger subset of transcripts than is currently recognized. To explore this possibility, we used gene microarray technology to identify and analyze large populations of cytosolic and cytoskeleton-associated mRNAs isolated from HL-60 promyelocytic cells. By combining traditional biochemical subcellular fractionation methods with massively-parallel gene profiling technology, we were able to ask the question: how many different eukaryotic mRNAs interact with the cytoskeletal network and what is their identity?

## Results

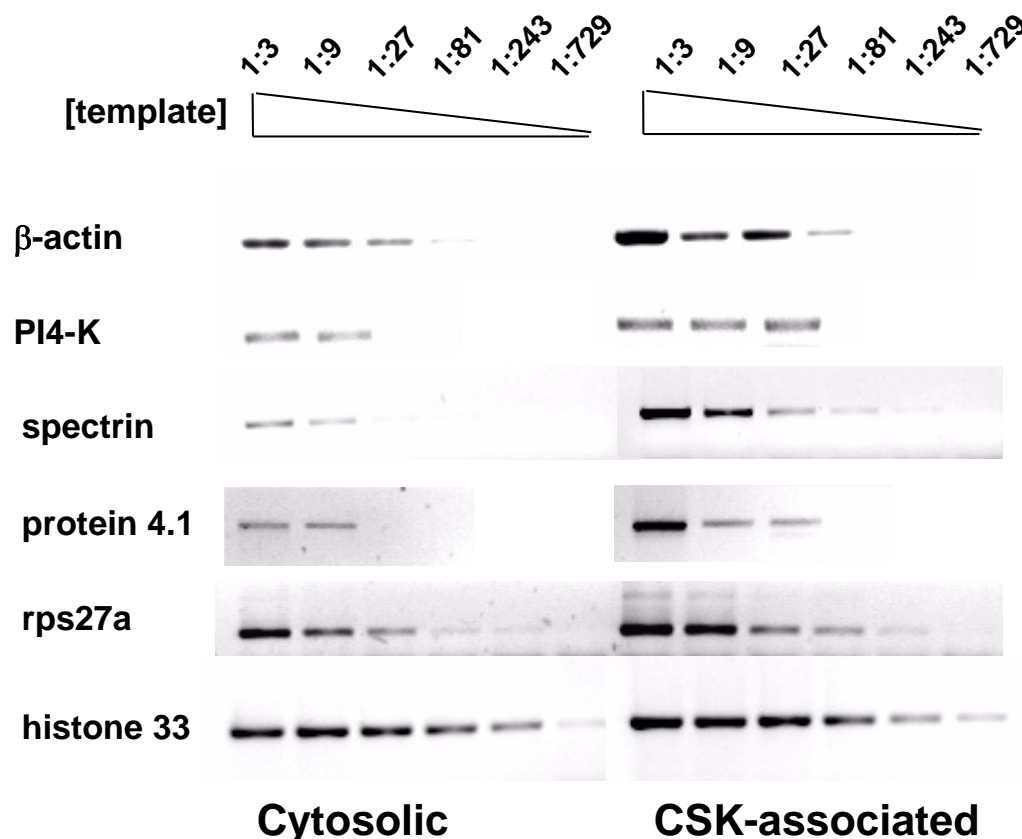
To investigate whether multiple mRNAs associate with the cytoskeleton, exponentially growing HL-60 promyelocytic cells were biochemically extracted to obtain fractions enriched for either cytoskeleton or cytosolic components. Briefly, cells were harvested and lysed in nonionic detergent to release the soluble, cytosolic fraction. Upon centrifugation, the pellet, containing cellular matrix, was resuspended in high salt buffer to release cytoskeleton-associated components. RNA was isolated from both fractions and hybridized to nylon filter DNA arrays that contained 5184 gene or EST sequences. After filtering out genes whose signal intensity was not significantly above background noise, 649 known genes remained. Analysis of these genes revealed a subset of transcripts that were enriched 2–15 fold in the cytoskeleton fraction relative to the cytosolic fraction (Table 1, top). Selected transcripts identified by microarray analysis, including  $\beta$ -actin, spectrin and phosphatidylinositol-4-kinase (PI4K), were sub-

sequently confirmed by semi-quantitative RT-PCR (Fig. 1).

Among the subset of transcripts that were found to be enriched in the cytoskeleton fraction were several mRNA species, such as those encoding  $\beta$ -actin mRNA and ribosomal protein subunits, that have previously been shown to be cytoskeleton-associated in other cell types [13,16]. In addition, we identified approximately 20 new members of the cytoskeleton-associated transcriptome, the majority of which encode structural proteins known to interact directly with cytoskeleton filaments, such as spectrin and protein 4.1 (Table 1). Spectrin is an integral component of the submembranous cytoskeleton that is required for maintenance of cell shape and mechanical stability of the surface membrane. Interactions between spectrin and actin filaments are stabilized by protein 4.1 and regulated by a variety of accessory proteins [17]. Protein 4.1 also may connect the protein translation apparatus to the cytoskeleton through interactions with a component of the eukaryotic translation initiation factor-3 complex, eIF3-p44 [18]. Interestingly, mRNAs for multiple proteins involved in translation, such as various ribosomal proteins, were enriched in the cytoskeleton fraction (Table 1). Other transcripts found to be cytoskeleton-associated in this study encode PI4K which associates with the cytoskeleton backbone of the focal adhesion [19] as well as thymosin  $\beta$ 4, an actin monomer sequestering protein that also contributes to control of cell motility and contractility [20].

To determine which specific class of cytoskeleton filaments is required for the localization of these mRNAs, cells were treated with latrunculin B (0.1  $\mu$ g/ml) for 30 min. Latrunculin B depolymerizes actin microfilaments and hence should release components that normally associate with the actin cytoskeleton. Western blotting of the biochemical fractions prior to RNA isolation confirmed that latrunculin B treatment resulted in a dramatic decrease in the amount of  $\beta$ -actin protein that was extracted in the cytoskeleton fraction. RT-PCR of RNA isolated from these samples revealed a shift of the cytoskeleton-associated transcripts to the cytosolic fraction upon exposure to latrunculin, indicating that these transcripts had been associated with the actin cytoskeleton in intact cells (Fig 2). In contrast, depolymerizing microtubules with nocodazole did not change the distribution of these transcripts between the cytosolic and cytoskeleton fractions (data not shown).

HL-60 promyelocytic leukemia cells have been widely used as a model system for the study of hematopoietic differentiation because they undergo terminal differentiation to one of several cell types of the myelo-monocytic lineage when stimulated with various pharmacological

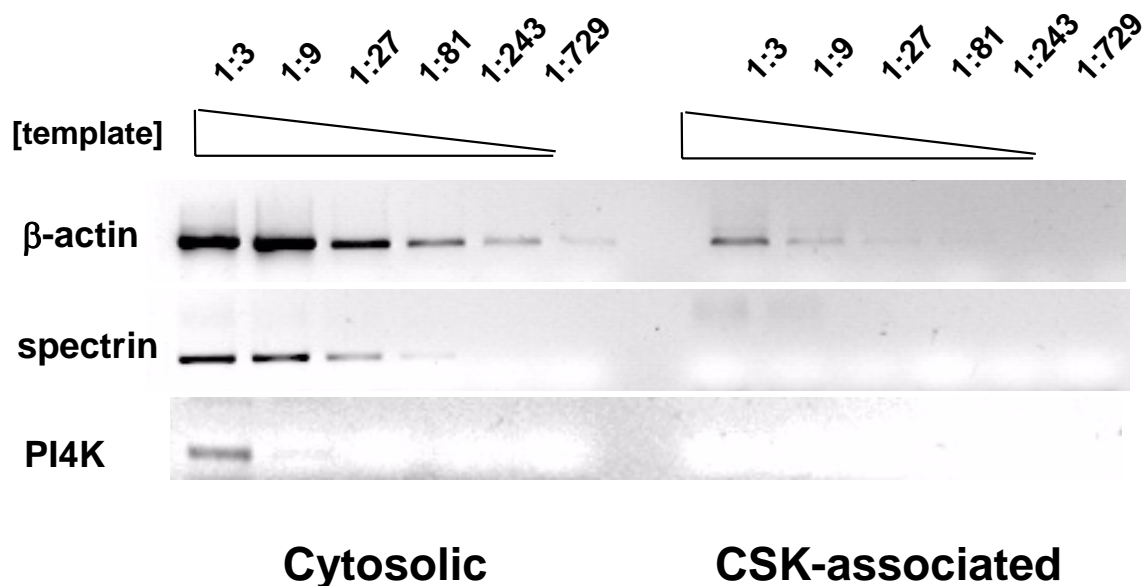
**Figure 1**

**RT-PCR analysis of mRNAs in the cytosolic and cytoskeleton-associated fractions.** Semi-quantitative RT-PCR of transcripts identified as cytoskeleton-associated by microarray hybridization. Serially diluted cDNA (1:3, 1:9, 1:27, 1:81, 1:243, 1:729 in ddH<sub>2</sub>O) from the cytosolic (left) and cytoskeleton-associated (right) fractions served as the template for PCR amplification. Other mRNAs that were confirmed using this approach include rps13, thymosin- $\beta$ 4, and glutathione peroxidase.

agents [21]. In particular, induction of macrophage differentiation by treatment of HL60 cells with TPA is accompanied by dramatic alterations in cell morphology and cytoskeleton structure. Precursor HL-60 cells are spherical and grow in suspension, whereas the differentiated macrophage-like cells become adherent and spread. To examine whether differentiation and reorganization of the cytoskeleton produces changes in mRNA distribution, macrophage differentiation was induced by the addition of 10 nM TPA to suspension cultures of exponentially proliferating HL-60 promyelocytic cells. Within 40 hrs of induction, the previously non-adherent cells (Fig. 3A) attached and spread on the tissue culture plastic (Fig. 3B). The serial extraction approach described above was used to separate cytosolic and cytoskeleton mRNA fractions

from these adherent cells. Spatial distribution profiles of mRNAs in differentiated HL-60 cells were obtained using microarrays and compared with profiles of the spherical precursor HL-60 cells.

Rather than comparing the entire transcriptome of undifferentiated versus differentiated cells as is commonly done [22], we were interested in how the two transcriptome compartments behaved upon differentiation and adhesion. We found that the terminally differentiated cells contained a greater number of cytoskeleton-associated transcripts than suspended precursor cells, consistent with the idea that the cytoskeleton plays a larger role in organizing RNAs in adherent cells (Table 1 bottom versus top, and Fig. 4). Approximately 22 unique mRNAs exhib-

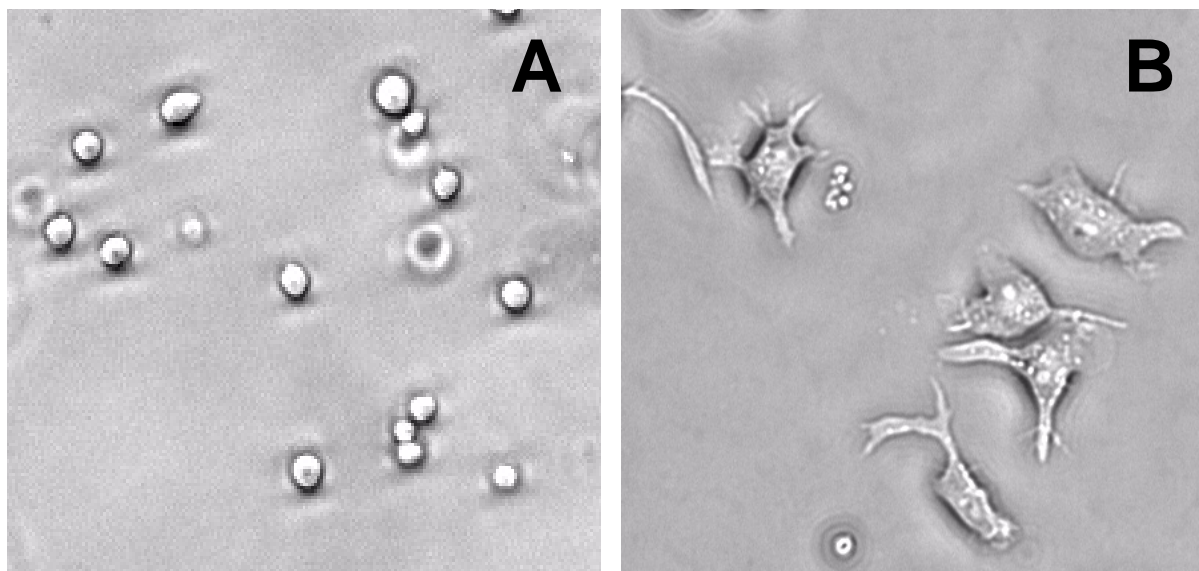
**Figure 2**

**Actin microfilament integrity is required for the cytoskeleton-association of transcripts.** HL-60 cells were treated with latrunculin B (0.1  $\mu$ g/ml) to depolymerize actin microfilaments. RT-PCR analysis reveals that cytoskeleton-associated transcripts shift to the cytosolic fraction upon latrunculin B treatment, indicating that actin filaments were critical in maintaining the cytoskeleton-localization of these transcripts. (A similar distribution and requirement for actin cytoskeleton integrity was observed in RT-PPCR studies of protein 4.1 and thymosin- $\beta$ 4)

ited 2-fold or more enrichment in the cytoskeleton-associated samples. A scatterplot of the ratios of cytoskeleton to cytosolic RNAs in the precursor and macrophage cells revealed a shift in the distribution of individual transcripts to the cytoskeletal compartment upon differentiation (Fig. 4). However, the majority of transcripts did not change their localization status as indicated by the fact that the ratios of most genes remained close to the diagonal in this plot.

To further characterize changes in the overall distribution pattern of mRNAs upon differentiation, we performed hierarchical cluster analysis of the four different RNA profiles representing the two spatial pools (cytosolic and

cytoskeleton) in the two cell types (undifferentiated and differentiated cells). Complete linkage clustering based on Euclidean distance [23] of these four sets of transcripts (cytosolic versus cytoskeleton transcripts in HL-60 cells in the presence or absence of TPA) revealed that the cytoskeleton-anchored population in the precursor HL-60 cells and those in the macrophage cells were most similar (Fig. 5). In other words, mRNAs within each distinct subcellular compartment clustered more closely together than those of the same cell type. Thus, the overall subcellular distribution of mRNAs is largely conserved across the two different cell types despite significant changes in cytoskeleton architecture, adhesive state and cell morphology.

**Figure 3**

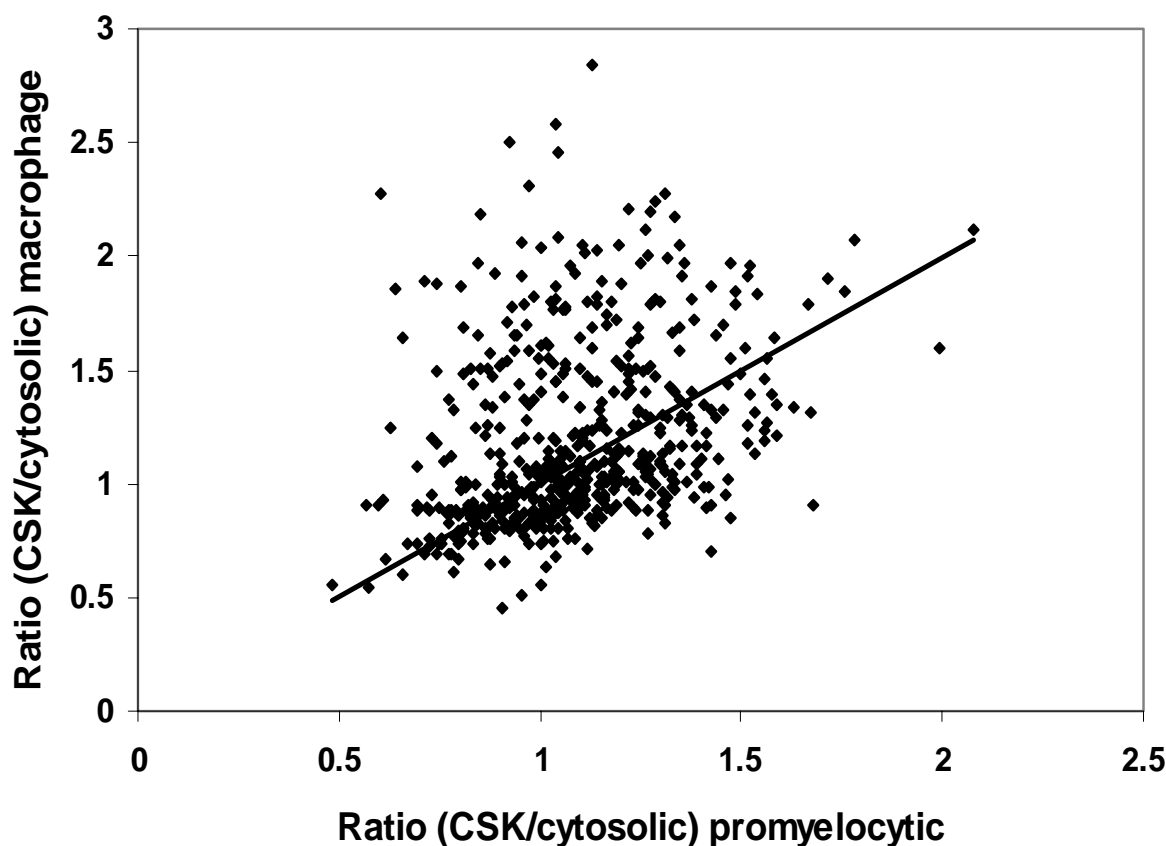
**Effects of TPA treatment on HL60 adhesion and morphology.** A) HL-60 promyelocytic cells remain spheroid when grown in suspension. B) Treatment with TPA (10 nM) induced macrophage differentiation, accompanied by cell adhesion and spreading on tissue culture plastic.

## Discussion

Interactions between mRNA and cytoskeleton filaments are responsible for the spatial distribution and localized function of several transcripts in somatic cells [15,16,24]. However, since only a small number of localized mRNAs have previously been uncovered, it is not known to what extent the association of RNAs with the cytoskeleton might be a more general mechanism of gene regulation. Our data suggest that while many mRNAs distribute equally between the cytosolic and cytoskeletal fractions, a specific set of transcripts associates preferentially with actin microfilaments.

Furthermore, this spatial distribution pattern was retained even after the round HL-60 cells were chemically induced to differentiate into macrophages that adhere and spread

on the culture substrate, a process that involves extensive alterations in cytoskeletal architecture and genome-wide changes in gene expression. Thus, the cytoskeleton-associated transcripts may encode a core group of proteins whose expression may be regulated at the post-transcriptional level through their association with the components of the actin cytoskeleton network. Consistent with this hypothesis, the majority of cytoskeleton-associated transcripts identified in this study encoded proteins that modulate cytoskeleton structure or adhesion. Spectrin and protein 4.1 form a complex with actin filaments [17,26] that may be important in signaling in addition to forming a physical link between the cytoskeletal network and the cell membrane [27]. Furthermore, PI4K activity has been shown to associate with the actin cytoskeleton by biochemical detergent extraction [28] and

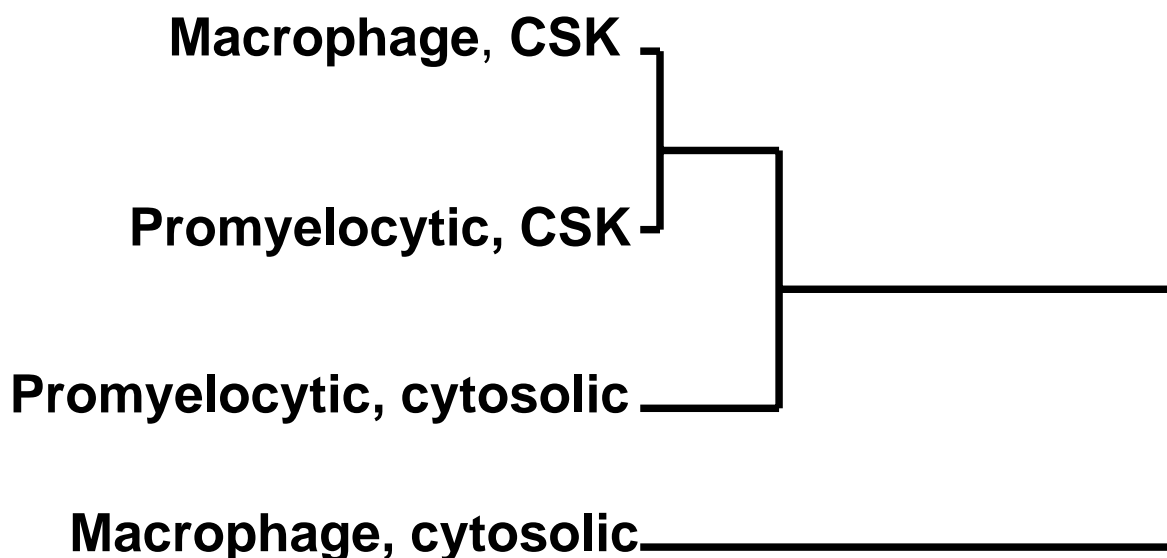
**Figure 4**

**Effects of macrophage differentiation on mRNA distribution.** Cytoskeleton-associated and cytosolic transcripts were isolated from HL-60 promyelocytic (precursor) cells and HL-60 differentiated to macrophage-like cells by phorbol ester induction. Microarray hybridization measured the relative amounts of 5184 mRNAs in the cytoskeleton and cytosolic populations of untreated or differentiated cells. Each of the 649 points represents the ratio of a specific transcript in the cytoskeleton-associated vs cytosolic fraction of the promyelocytic or differentiated cell type. Transcripts whose signal intensity was not above the background noise level were removed prior to analysis.

is thought to specifically localize with  $\alpha 3 \beta 1$  integrin [29]. PI4K catalyzes the production of phosphatidylinositol-4-phosphate, an intermediate in multiple phosphatidylinositol signaling pathways which itself can interact with actin binding proteins to regulate microfilament polymerization [30]. Our work now suggests that the mRNAs of these proteins are all positioned in close association with the actin cytoskeleton to permit efficient localization of their protein products.

Given the limited sensitivity of DNA arrays, it is possible that the cytoskeleton may play a role in the localization and regulation of additional cellular transcripts that could not be detected with the approach described here. Abun-

dant mRNA species may target only a miniscule fraction of mRNA molecules to the cytoskeleton; this may be the functionally important fraction but its relevance would not be revealed using this method. On the other hand, mRNAs of lowly expressed genes may be entirely associated with the cytoskeleton but may fall below the limits of microarray detection. For the majority of gene transcripts, the relationship between mRNA localization and translational activation remains unclear. It is therefore possible that although only a small fraction of a particular transcript associates with the cytoskeleton, these localized molecules might actually represent the actively translated subpopulation.

**Figure 5**

**Hierarchical cluster analysis of cytosolic and cytoskeleton-associated transcriptome profiles in promyelocytic and differentiated HL-60.** Complete linkage clustering by Euclidean distance was performed to compare the four sets of transcripts (cytosolic versus cytoskeleton transcripts in HL-60 cells in the presence or absence of phorbol ester). The cytoskeleton-associated populations in promyelocytic and phorbol ester-induced differentiated cells cluster most closely together, suggesting that the spatial distribution of cytoskeleton-associated transcripts may be largely conserved across different cell types.

This naturally raises the question of whether the cytoskeleton-associated transcripts are differentially expressed in promyelocyte and macrophage cells. Tamayo *et al.* have previously monitored gene expression at various time points following phorbol ester-induced differentiation in the same HL-60 cell system [22]. Thirteen of the 22 transcripts we identified as cytoskeleton-associated were also assayed in that study by Tamayo, *et al* [22]. Only two of these transcripts—alpha spectrin and pim-1—were found to vary significantly, in that the expression of both genes was undetectable in the promyelocytic cell type and increased dramatically upon differentiation to the macrophage cell type. Future work in this area might address the potential

relationship between cytoskeleton-localization and expression of these transcripts.

As a mechanosensitive scaffold that controls a large set of regulatory proteins, the actin cytoskeleton is critically poised to modulate a variety of cellular processes, including protein synthesis, cell survival, migration and proliferation [14,31,32]. One function of the cytoskeleton in gene regulation appears to be the spatial organization of transcripts and possibly their translation within the intracellular environment. Our work demonstrates that a subset of gene transcripts interacts with the actin cytoskeleton and that this subpopulation includes a number of structural and cytoskeleton-modulating proteins as well



**Table 1: Cytoskeleton-associated mRNAs identified in HL-60 cells.**

CSK-associated transcripts in promyelocytic HL-60		
<i>Title</i>	<i>Ratio</i>	<i>Abbreviation</i>
actin, beta	5.2	ACTB
protein 4.1 isoform	3.7	
thymosin, beta 4	2.8	TMSB4X
ribosomal protein S13	2.4	RPS13
ribosomal protein S27a	2.2	RPS27A
phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	2.0	PIK4CA
CSK-associated transcripts in macrophage-like HL-60		
<i>Title</i>	<i>Ratio</i>	<i>Abbreviation</i>
Protein 4.1 isoform	15.6	
Mnt (Max-binding protein)	3.6	MNT
actin, beta	3.2	ACTB
spectrin, alpha	2.7	SPTA1
claudin 1	2.7	CLDN1
protein kinase, interferon-inducible double stranded RNA dependent	2.7	PRKR
GTP-binding protein ragB	2.5	RAGB
protein tyrosine phosphatase type IVA, member 2	2.5	PTP4A2
glutathione peroxidase 1	2.5	GPX1
cadherin 11 (OB-cadherin, osteoblast)	2.4	CDH11
histidyl-tRNA synthetase	2.3	HARS
pim-1 oncogene	2.2	PIMI
phosphoglycerate mutase 1 (brain)	2.2	PGAM1
calcium channel, voltage-dependent, alpha 2/delta subunit 2	2.2	CACNA2D2
solute carrier family 21 (organic anion transporter), member 3	2.2	SLC21A3
phosphofructokinase, muscle	2.1	PFKM
rearranged L-myc fusion sequence	2.1	RLF
ribosomal protein S6	2.0	RPS6

Cytoskeleton-associated and cytosolic transcripts were isolated from both cell types and identified by microarray analysis. Transcripts that were found to be greater than two-fold enriched in the cytoskeleton fraction of the promyelocytic or macrophage cells are listed. (ratio= expression level in cytoskeleton fraction/expression level in cytosolic fraction)

as molecules involved in protein translation. This finding is consistent with the hypothesis that cellular evolution resulted from self-assembly of molecular scaffolds, like the actin cytoskeleton, that provide simultaneous structural and biochemical functions, including orienting mRNA transcripts and facilitating their translation into proteins that self-assemble into the very scaffolds to which they associate [33].

In summary, this work extends our view of post-transcriptional control of gene expression by revealing that multiple transcripts involved in cellular regulation associate with the actin cytoskeleton. The subcellular localization of the majority of these transcripts had not been previously investigated. It appears that information about the spatial context of an mRNA species, in addition to knowledge of its temporal expression pattern, may be necessary for a more complete view of gene regulation. Thus, a combina-

tion of other biochemical fractionation techniques (e.g., isolation of microtubule or intermediate filament cytoskeleton compartments, nuclear matrix, etc.) with massively parallel gene profiling technology may provide additional insights into cellular regulation.

## Conclusions

In this work, we set out to explore the extent to which somatic cell transcripts associate with the cytoskeleton and to identify the specific transcripts that might be regulated through cytoskeleton localization. A set of 22 transcripts, encoding structural proteins, cytoskeletal proteins and components of the translation machinery, was shown to be highly enriched in the cytoskeleton-associated population of HL-60 cells. Intact actin microfilaments were required for the localization and/or anchoring of these transcripts. In addition, this study emphasizes the value of using subcellular fractionation to expand the range of

genome-wide technologies by providing information about the spatial distribution of a transcript, in addition to its expression level.

## Methods

### Cell culture conditions

Suspended HL-60 promyelocytic leukemia cells were cultured in IMDM (Clonetics) supplemented with 10% fetal bovine serum (Hyclone). Cells were seeded in tissue culture flasks at a density of  $3 \times 10^5$  cells/ml and expanded when the culture reached  $3 \times 10^6$  cells/ml. To induce macrophage differentiation, 10 nM phorbol ester (TPA) was added to growth cultures at a cell density of  $1.5 \times 10^6$  cells/ml, 40 hrs prior to harvest. To depolymerize actin microfilaments, cells were treated with 0.1  $\mu$ g/ml latrunculin B (Cytoskeleton, Inc.) 30 min prior to harvest.

### Cell harvest and serial extraction

Subcellular fractionation of RNA into cytoskeleton-associated and cytosolic fractions was performed as previously described [15,34]. In brief, HL-60 cells were harvested by centrifugation and resuspension in buffer A (10 mM Tris pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM  $MgCl_2$ ) with 0.1% Igepal nonionic detergent (Sigma). The minimal concentration of nonionic detergent required to release cytosolic components without disrupting nuclei was determined by measuring lactate dehydrogenase activity as a marker for release of cytosol. The detergent concentration that released <90% of the lactate dehydrogenase activity in the cytosolic fraction was chosen for all subsequent studies. TPA-treated cells become adherent and were harvested at 40 hr by scraping in buffer A, pelleting the cells, and resuspending in buffer A plus 0.1% Igepal. After incubating for 10 min on ice, the samples were pelleted by centrifugation (1000 g) and the supernatant containing "cytosolic" components was collected. The pellet was resuspended in high salt buffer B (10 mM Tris pH 7.6, 0.25 M sucrose, 200 mM KCl, 5 mM  $MgCl_2$ , 0.5 mM  $CaCl_2$ ) and incubated 10 min on ice to disrupt the cytoskeleton and release associated components. Nuclei and internal membranes were removed by centrifugation (1500 g) and the supernatant containing "cytoskeleton-associated" components was collected. RNA was isolated from the cytosolic and cytoskeleton-associated fractions using RNeasy miniprep column and reagents (Qiagen). As an internal control for the RNA isolation procedure, an exogenous RNA that was generated by *in vitro* transcription from a pBluscriptII plasmid was added to the fractions prior to RNA isolation. RT-PCR analysis confirmed that the exogenous pBluII RNA was equally distributed between the cytosolic and cytoskeleton-associated fractions.

### Microarray hybridization and analysis

Small samples (2  $\mu$ g) from each RNA fraction were reverse transcribed and radiolabeled (2  $\mu$ g oligo dT, 1x First Strand buffer, 5 mM DTT, 75 U SuperscriptII [Life Technologies], 20 mM dNTP mix containing dATP, dGTP, dTTP and 100 mCi  $^{32}P$ -dCTP (ICN Radiochemicals). Probes were purified by passage through a Bio-Spin6 chromatography column (Bio-Rad). A Research Genetics gene filter (GF201) was prehybridized in MicroHyb solution (Research Genetics) containing 5  $\mu$ g Cot-1 DNA (Life Technologies) and 5  $\mu$ g poly dA at 42°C for at least 2.5 hr. The probe was denatured by heating to 95°C for 3 min before adding to the prehybridization solution. Hybridization was carried out overnight at 42°C in a hybridization roller oven. Membranes were washed twice in 2x SSC, 1% SDS at 50°C for 20 min and once in 0.5x SSC, 1% SDS at 55°C for 15 min. After washing, the filter was placed on moist filter paper, sealed in plastic wrap and exposed to a phosphor imaging screen for 1–2 days. Images were acquired using the GeneStorm phosphor imaging system (Molecular Dynamics). Filters were stripped by placing in boiling 0.5% SDS with shaking for 1 hr. Each sample probe was sequentially hybridized to the same human gene filter to control for variation between filters. The Pathways 4.0 (Research Genetics) software package was used for normalization and initial comparisons. Additional analysis was performed using Matlab 5.3 (Mathworks) and Clustan 4 (Clustan Graphics). Comparison of multiple scans of the same sample revealed that low intensity spots (intensity < 1) were variable due to background noise; these were not included in subsequent analysis.

### Western blotting

HL-60 cells were harvested and serial extractions were performed as described above. Equal volumes of each sample (cytosolic and cytoskeleton-associated components) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad) and immunoblotted with primary antibody to  $\beta$ -actin and detected using horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) and SuperSignal West Dura (Pierce) as a chemiluminescent substrate.

### RT-PCR

Semi-quantitative reverse transcription (RT)-PCR was used to confirm the relative abundance of specific mRNA species in the cytosolic and cytoskeleton-associated populations. In addition, the abundance of histone 33, a transcript that distributed equally between the cytosolic and cytoskeleton fractions in microarray analysis, was confirmed. cDNA was synthesized from each sample by incubating 1  $\mu$ g RNA sample with 0.5 mM each dNTP, 0.25  $\mu$ g/ml random hexamer (Boehringer Mannheim), 10 U RNase inhibitor, and 10 U OmniScript reverse tran-

scriptase in OmniScript RT Buffer (Qiagen). Two microliters of serially diluted template cDNA (1:3, 1:9, 1:27, 1:81, 1:243, 1:729 in ddH<sub>2</sub>O) was used for each PCR reaction in a total volume of 24 µl containing 10 pmol of each primer, 0.2 mM each dNTP and 2.5 U Taq DNA polymerase in Qiagen PCR buffer. PCR cycling conditions were 4 min at 94°C and then 24, 26 or 30 cycles of 45 sec at 94°C, 45 sec at 55–63°C and 1 min at 72°C. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized in a FluorImager (Molecular Dynamics, Sunnydale, CA). To determine conditions of log-linear amplification (with slope = 1), the PCR products from each dilution of cDNA template were quantified using NIH Image software.

### Authors' contributions

AB carried out all experiments described in this work and drafted the manuscript. SH participated in the design of the study and assisted in analysis of the microarray data. DEI participated in the design and coordination of the study. All authors read and approved the final manuscript.

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